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14. ABSTRACT

A central tenet of the field of bone metastases is that the bone microenvironment supplies factors, such as TGF-beta, stimulating prostate cancer cell signaling and altering their phenotype. TGF-beta signaling in cancer is however complex and can lead to the activation of numerous genes. We have identified many of these genes by microarray analysis and have validated the gene reported here. Of these, PMEPA1 as the most highly upregulated gene. We have cloned the PMEPA1 promoter and full-length gene and have begun promoter analysis of the TGFβbetaresponse element. We are in the process of overexpressing PMEPA1 in prostate cancer lines. In vivo experiments are in progress to determine the effect of a TGFbeta RI kinase inhibitor, SD-208, on the development and progression of prostate cancer metastases to bone due to PC-3, LuCAP and C42B prostate cancers.

15. SUBJECT TERMS
Bone metastases, prostate cancer, TGF-beta, PMEPA1

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Introduction

Prostate cancer has a propensity to grow in the skeleton and cause significant morbidity. Once housed in bone, prostate cancer is incurable. Bone is a rich storehouse of growth factors, which stimulate signaling in metastatic cancer cells. Bone-derived TGFβ increases tumor secretion of factors that activate bone remodeling, fueling a vicious cycle, which drives the growth and survival of prostate bone metastases. In prostate cancer cells, TGFβ signals through two receptor subunits and, further downstream, p38 MAP kinase. Hypothesis: *TGFβ mediates prostate cancer metastases to bone via p38 MAP kinase pathway. TGFβ and/or p38MAP kinase signaling inhibitors will reduce the development and progression of prostate cancer bone metastases to bone.* Two orally active inhibitors of these serine/threonine kinases will be tested in an animal model of prostate cancer bone metastases. We propose three Specific Aims. Aim 1: To test a TGFβRI kinase inhibitor and a p38 MAPK inhibitor against three human prostate cancer models of skeletal metastasis in mice. Aim 2: To determine the molecular targets of these inhibitors in prostate cancer cells *in vitro* and test their impact on tumor growth and bone metastases *in vivo*. Aim 3: To test the efficacy of combined TGFβRI and p38 MAP kinase inhibitors against three prostate cancer models *in vivo*.

Body

BACKGROUND. The skeleton is a major site of metastasis by advanced prostate cancer. Last year 220,900 cases of prostate cancer were diagnosed in the United States, where it is now the most commonly diagnosed cancer and the second most common cause of cancer mortality in men, with 28,900 deaths (Crawford, 2003). One fourth of diagnosed patients will die from the disease, the majority of them with metastases to the skeleton. Once cancer becomes housed in bone, it is incurable. The average survival from time of diagnosis of skeletal metastases in prostate cancer patients is 40 months. When prostate tumor cells metastasize to the skeleton, the most common response is osteoblastic: characterized by net formation of disorganized new bone, which results in fractures, severe and intractable bone pain, and nerve compression. Metastasis to bone thus causes prolonged, serious morbidity for many prostate cancer patients. Treatment to prevent or halt the progression of bone metastases (Reddi et al, 2003; O'Keefe and Guise, 2003). would increase survival and improve quality of life for men with prostate cancer

Transforming growth factor-β in cancer is a two-edged sword. TGFβ is a growth inhibitor and a tumor suppressor at early stages of the oncogenic cascade. However, advanced cancers often lose the growth inhibition by TGFβ but continue to respond to the factor. The net effect is that TGFβ is a metastasis enhancer for advanced cancers. Since bone is a major source of active TGFβ, the factor plays a crucial role in the vicious cycle of bone metastases. Blockade of the TGFβ pathway effectively decreases metastases in several animal models (Yin et al, 1996; Muraoka et al, 2002; Yang et al, 2002).

Transforming growth factor-β in bone is released from mineralized matrix in active form by osteoclastic resorption (Dallas et al, 2002), which is very prominent in prostate cancer metastases. TGFβ acts on tumor cells to increase the secretion of factors that inappropriately stimulate bone cells (Chirgwin & Guise, 2003a,b). The interactions

between bone and cancer constitute a vicious cycle, which enhances skeletal metastases (Mundy, 2002). Extensive data show that TGFβ is a major bone-derived factor responsible for driving the vicious cycle of cancer metastases in bone. TGFβ increases tumor secretion of factors such as endothelin-1, IL-6, IL-11, PTHrP, and VEGF. These factors stimulate both osteoblastic synthesis of disorganized new bone and osteolytic destruction of the skeleton adjacent to tumor cells. The cellular and molecular components of the vicious cycle between tumor and bone offer opportunities for therapeutic intervention to decrease skeletal metastases (Coleman, 2002; Guise & Chirgwin, 2003a). TGFβ in particular is an important target for intervention against prostate cancer skeletal metastases.

Therapy to block TGFβ signaling in bone metastases. Previous work has demonstrated the effectiveness of TGF-beta inhibition to decrease metastases, but these experiments have used protein-based treatment or ex vivo manipulations of the tumor cells (Yin et al, 1996; Muraoka et al, 2002; Yang et al, 2002). Orally active small-molecule inhibitors of the TGFβ pathway would be much more practical. This proposal will test two inhibitors of serine/threonine kinases. The first directly targets the TGFβ receptor kinase. The second targets p38 MAP kinase, which is a major downstream effector of TGFβ signaling in cancer cells. Both targets are serine/threonine kinases. Our preliminary data show that inhibition of TGFβ signaling is effective in an animal model of cancer bone metastases. The work proposed will test the two serine/theronoine kinases inhibitors in animal models of human prostate cancer in bone: one in which the response is osteolytic, two others in which it is osteoblastic. The experiments proposed will rapidly provide the preclinical data necessary for these two drugs to be placed in clinical trails for prostate cancer bone metastases.

Hypotheses: 1) TGFβ mediates prostate cancer metastases to bone via p38 MAP kinase. Specific serine/threonine kinase small-molecule inhibitors of the type I TGFβ receptor kinase and of p38 MAP kinase will reduce the development and progression of prostate cancer metastases to bone, due to either osteoblastic or osteolytic diseases. 2) Orally active inhibitors of these serine/threonine kinases will be effective in animal models of prostate cancer bone metastases to decrease metastases and tumor burden and to increase survival. 3) The two drugs may be more effective in combination than singly, if p38 MAP kinase also mediates TGFβ-independent metastatic functions. 4) Specific targets of TGFβ signaling in prostate cancer cells contribute directly to the bone phenotype of metastases. One such factor may be the type I membrane protein PMEPA1, which is regulated by TGFβ and expressed by prostate cancers. 5) Expression of PMEPA1 on the surface of cancer cells will increase the development and progression of prostate cancer metastases to bone.

Specific Aim 1: To determine the effect of TGFβRI kinase or p38 MAPK blockade separately against 3 human prostate cancer models of skeletal metastasis in mice (hypotheses 1 & 2).

Results and Progress: This experiment is in progress with respect to the TGFβRI kinase inhibitor. We are testing the effects of the TGFβRI kinase, SD-208, on the development and progression of bone metastases due to PC-3 prostate cancer. This aim has taken

longer than originally planned because we had to determine long-term pharmacokinetics for drug delivery in the food. We have pharmacokinetic data that 50 and 100 mg/kg of SD-208 added to food result in drug levels that were effective in our mouse model of breast cancer metastases to bone. The p38MAPK inhibitor experiments have not been started as our pilot experiment showed that drug treatment appeared to be toxic for the mice: they developed fragile bones. However, we plan to test the p38MAPK inhibitor in PC-3 tumor-bearing mice in the next grant year, once we have established the all the toxicities associated with this compound.

Specific Aim 2: To determine the molecular targets of the inhibitors in prostate cancer cells in vitro by gene array analysis (hypothesis 4). The role of an already-identified target of TGFβ, PMEPA1, will be tested in the animal models by overexpressing it in 2 prostate cancer cell lines (hypothesis 5).

Results and Progress: The majority of the work from year 1 has been performed on this aim. Gene array targets of TGFβ on PC-3 prostate cancer were validated by quantitative real-time PCR and are described below.

Specific Aim 3: To test the efficacy of combined TβRI and p38 MAPK inhibitors against 3 prostate cancer models in vivo (hypothesis 3).

Results and Progress: This aim has not been started and is planned for the last year of the proposal.

Results:
Validation of the micro-array experiment using qRT-PCR

Method

PC-3 cells were grown in cell culture dishes until they reach near confluency and were starved for 24H in F12-K basal medium. The cell monolayer was rinsed twice with PBS and the cells were further cultured in F12-K medium containing or not containing TGF-β1 (5ng/mL) for different amount of time (0, 1, 2, 4, 8, 12, 24 and 48H).

The conditioned media were collected and supplemented with anti-proteases (aprotinin and leupeptin, 2µg/mL final concentration). The solutions were centrifuged (1,000g, 5min, 4°C) to remove cell debris and the supernatant were aliquoted and frozen at -80°C for further protein analysis.

The cell monolayers were rinsed with PBS and trypsinized (2mL Trypsine/EDTA, 3min, 37°C). Trypsinization was stopped by adding 8mL of ice-cold complete F12-K. Cells were centrifuged (800g, 5min, 4°C) and the pellets were washed twice in ice-cold PBS. The total RNA was extracted from cell pellets (RNeasy lipid tissue kit, Qiagen) and treated with DNase I to avoid DNA contamination (RNase free DNase set, Qiagen). RNA was used for cDNA synthesis (SuperScript ™ II reverse transciptase kit, Invitrogen). The cDNA were used as template in a quantitative real-time PCR (QuantiTect SYBR green PCR kit, Qiagen) using BioRad MyiQ thermocycler (annealing temperature 58°C).

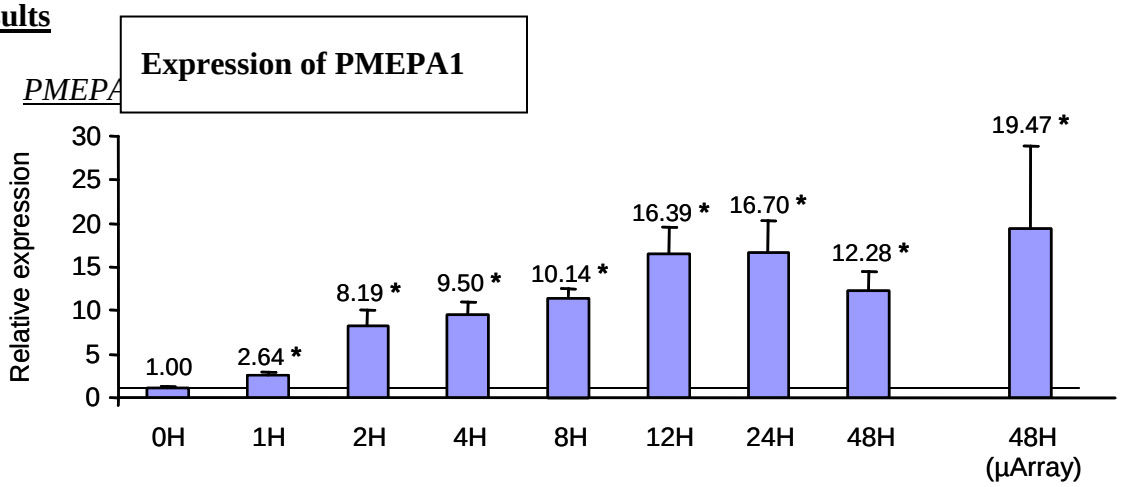
Similar real-time PCR were run with the cDNA obtained from the PC-3 cells, treated or not treated with TGF-β (5ng/mL, 48H) and used for the micro-array experiment.

Primer used for the qRT-PCRs were chosen for genes selected from the micro-array analysis (PMEPA1, MMP-13, ADAM19, THBSP1, PTHrP, CTGF, VEGF) or from the literature (IL-6, IL-11, Cyr61, VEGF121, VEGF165). Endogenous genes human RpL32 (ribosomal protein L32) and HPRT (hypoxanthine phosphoribosyltransferase 1) were used to normalized the results. Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

All conditions were run as a triplicate. The real-time threshold values (Ct) were analyzed using the ΔΔCt method, where the amount of mRNA is calculated as $2^{-\Delta\Delta Ct}$. The untreated cells at the same time-point were used as a calibrator at 1.0. L32 and HPRT were both used as endogenous genes and gave similar results. Data shown were calculated using L32 and values represent the means ± SD of the triplicate. Statistical significance was calculated using an unpaired, one-tailed Student *t* test (*, $P<0.05$ when compared to the untreated cells at 0H).

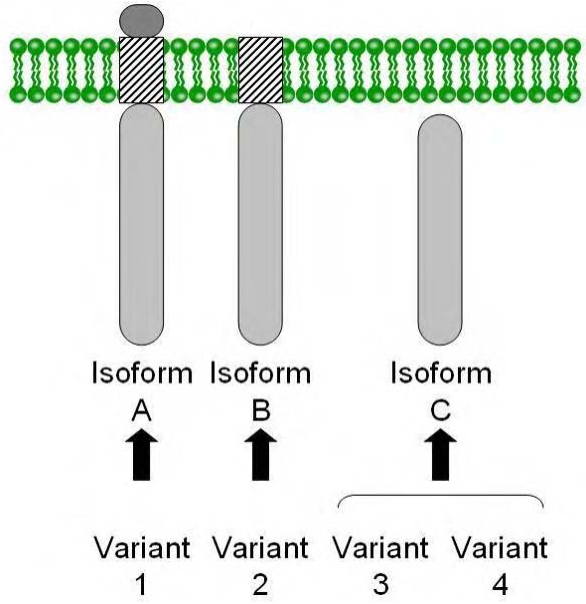
Gene	Forward (5'→3')	Reverse (5'→3')
L32	CAG GGT TCG TAG AAG ATT CAA GGG	CTT GGA GGA AAC ATT GTG AGC GAT C
HPRT	CCT CCG CCT CCT CCT CTG	CCT GGT TCA TCA CTA CTA ATC ACG
PTHrP	ACG GCG ACG ATT CTT CCT TC	CCA CCT TGT TAG TTT CCT GAG TTA
CTGF	GCT ACC ACA TTT CCT ACC TAG AAA TCA	GAC AGT CCG TCA AAA CAG ATT GTT
Cyr61	CCT CGG CTG GTC AAA GTT AC	AGG CTC CAT TCC AAA AAC AG
PMEPA1	AGA ACA CTC CGC GCT TCT TA	AGA ACA CTC CGC GCT TCT TA
MMP-13	AAC ATC CAA AAA CGC CAG AC	GGA AGT TCT GGC CAA AAT GA
VEGF(all)	AAG GAG GAG GGC AGA ATC AT	CAC ACA GGA TGG CTT GAA GA
VEGF165	GAA AAT CCC TGT GGG CCT TG	GTC ACA TCT GCA AGT ACG TTC G
VEGF121	CCC ACT GAG GAG TCC AAC AT	TGC GCT TGT CAC ATT TTT CTT G
IL-6	GAA AGC AGC AAA GAG GCA CT	TTT CAC CAG GCA AGT CTC CT
IL-11	TGA AGA CTC GGC TGT GAC C	CCT CAC GGA AGG ACT GTC TC
ADAM19	CAG TCA GGT GGT GGA ATG TG	ATT GCA GCA GGG GTT GTT AC
THBSP1-A	CAG TCA GGT GGT GGA ATG TG	ATT GCA GCA GGG GTT GTT AC
THBSP1-B	TTT CTC AGA ACA GCG GGA CT	TGT TGA TCA CCT TTC GCT TG
PMEPA1 var1	AAA CCC GAT CTC CTT GGA CT	AGC CTC TGC CGC TAG CTT
PMEPA1 var2	AAA CCA GGC AAT GGC GGA G	GAC CGT GCA GAC AGC TTG TA
PMEPA1 var3	AAA CCA GCG GAG CTG GAG T	GAC CGT GCA GAC AGC TTG TA
PMEPA1 var4	CAA GCC TCC TGG TCT TTC TG	GCT AGA CCA GAG CGA ATT CA

Results



Fold change measured by the micro-array: ×23.15 *vs* untreated cells.

PMEPA1 expression is significantly increased, rapidly after TGF-β treatment (within 1H) and reaches a maximum at 24H. Cycloheximide will be added to the medium to determine whether the induction is direct.



PMEPA1 Variants

Four different mRNA variants result from the transcription of the PMEPA1 gene, after alternative splicing. These variants code for 3 different isoform of the protein which functions are unknown. Real-time PCR was performed on the cDNA of PC-3 cells treated or not treated with TGF-β (5ng/mL) for 24H, using pairs of primers specific for the different variants.

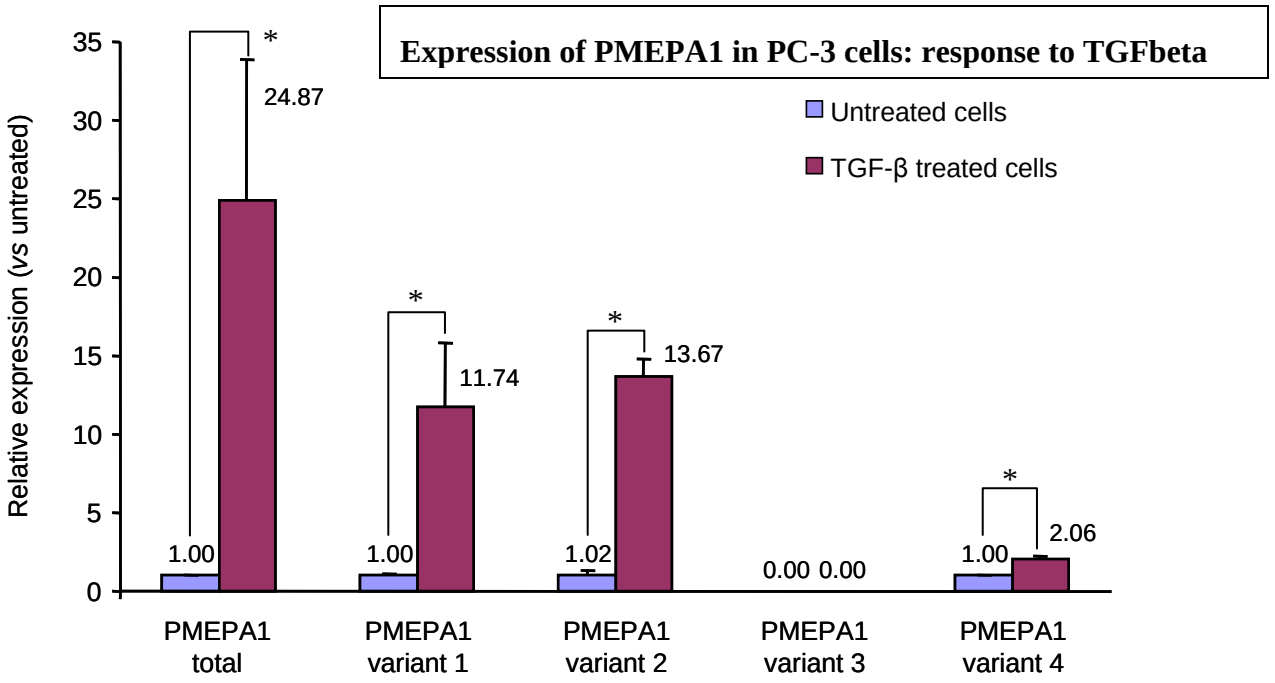
These results indicate that variant 1, 2 and 4 are expressed in PC-3 cells (Shown below). The expression of these mRNA is increased by TGF-β. No PCR product could be detected for the variant 3, using 2 different pairs of primers, suggesting that it is not produced. The ΔCt values of the different variant indicate that:

1) in untreated cells: variant 4 > variant 2 > variant 1
2) in TGF-β treated cells: variant 2 > variant 4 > variant 1

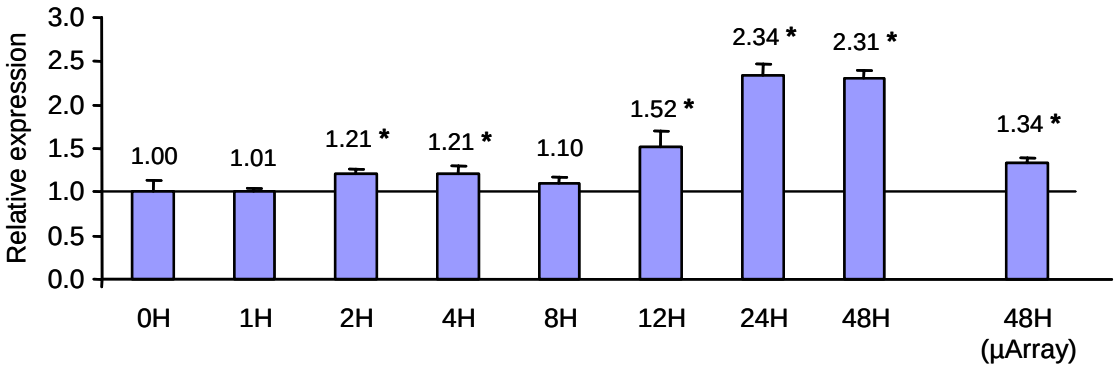
The membrane anchored protein may be predominant form in TGF-β treated cells. But, once again, these results must be further confirmed using amplification products to assess the reaction efficiency and to realize a standard curve.

We have cloned the promoter for PMEPA1 and have started analysis of TGFβ regulation of this promoter. We are also in the process of knock-down or overexpression of PMEPA1 in PC-3 prostate cancer.

Link to PubMed Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=56937.



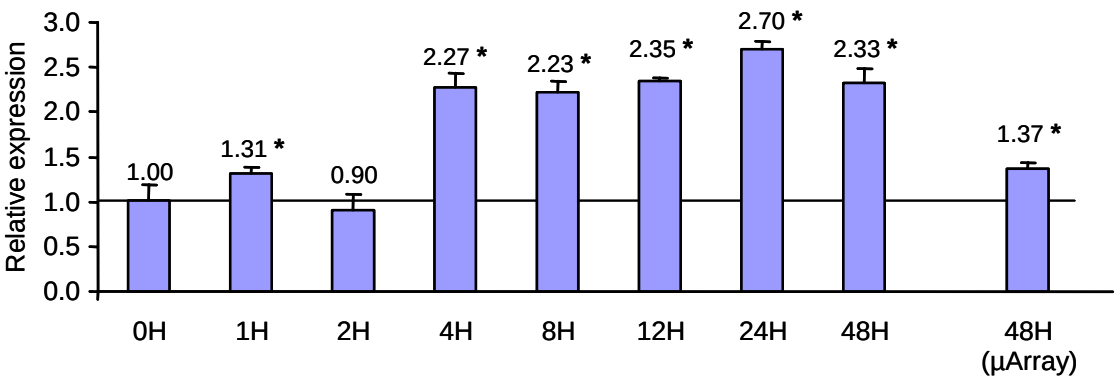
MMP-13 (collagenase 3)



Fold change measured by the micro-array: ×3.40 *vs* untreated cells.

TGF-β induces an increase of MMP-13 transcription after 12H and that reach a maximum at 24-48H. This late activation suggests that it is an indirect process due to another gene activated by TGF-β. This will be determined using cycloheximide analysis. The role of MMPs in bone metastasis is known to be important (Kang et al, 2003; Lynch et al, 2005) and should prove to be a worthy target for TGF-β inhibition.

ADAM19 (A Disintegrin And Metalloprotease Domain 19 or meltrin β)

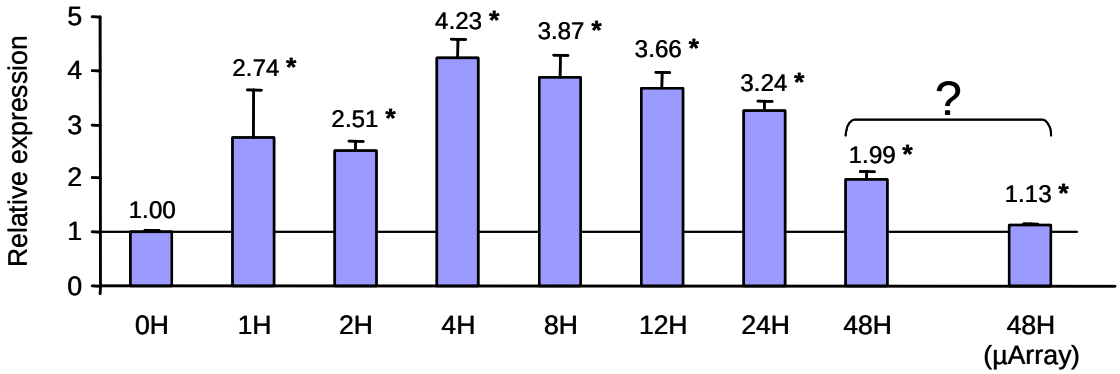


Fold change measured by the micro-array: ×2.73 *vs* untreated cells.

Four hours after the beginning of TGF-β treatment, ADAM19 transcription is stably upregulated, so it may then be a direct target of the TGF-β pathway. Little is known about the regulation of ADAM19 by TGF-β. Two different forms of ADAM19 mRNA result from alternative splicing. Some ADAM proteins (i.e., ADAM12 also know as meltrin α, ADAM17 also know as TACE) have been shown to be associated with osteoclastogenesis. Some ADAM proteins are important for the TNFα shedding (Zheng *et al.* 2004. JBC. 279:42898) and can then release RANKL from the membrane, regulating osteoclastogenesis (Lum *et al.*1999. JBC. 274:13613). Further study of the role of ADAM19 in the osteoclastogenesis due to the osteolytic PC-3 and to compare with osteoblastic prostate cancer cell lines (i.e. C4-2B) is justified by these studies.

Link to PubMed Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=8728

Thrombospondin-1 (or TSP1)

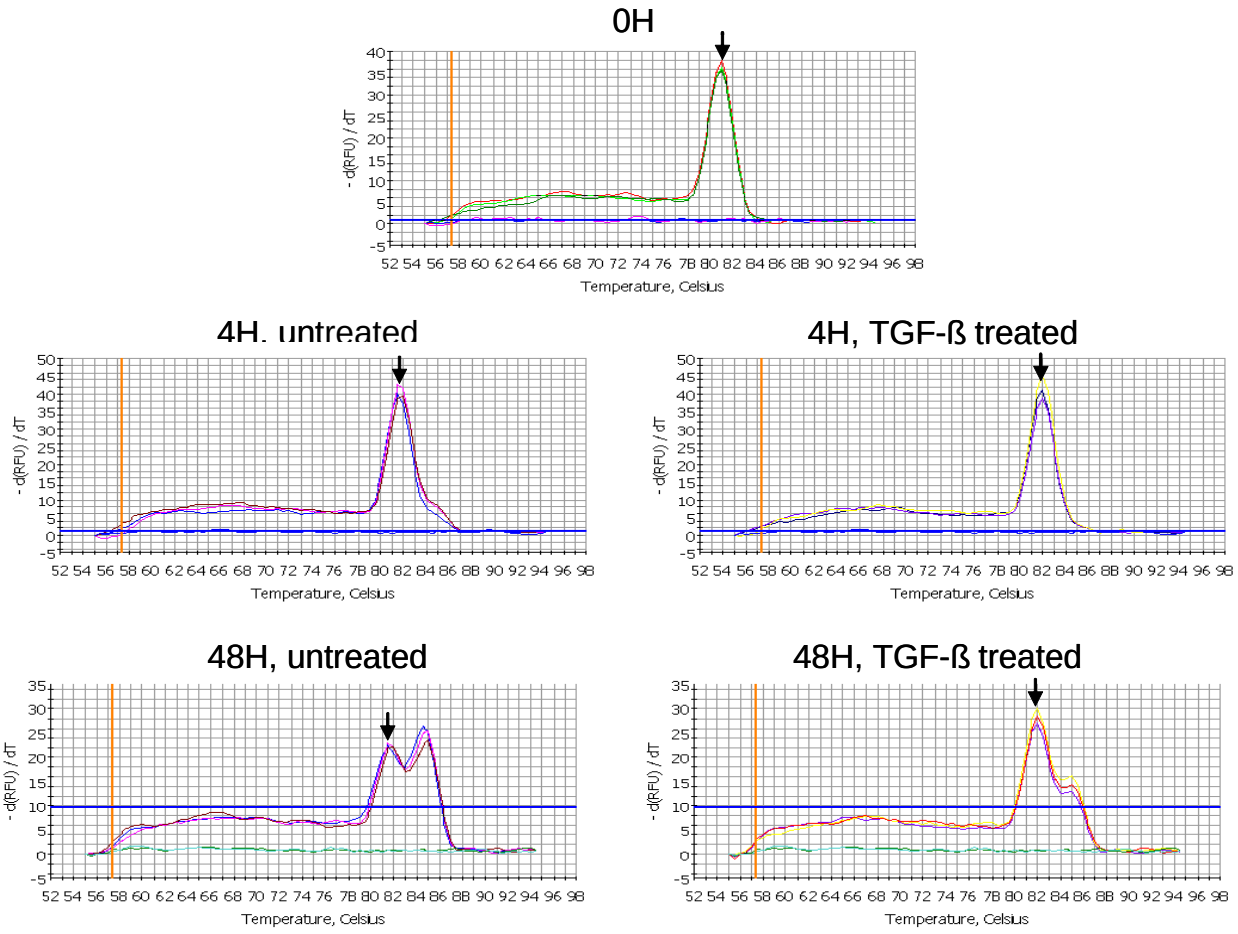


Fold change measured by the micro-array: ×3.81 or ×2.27 (pending on the probe) *vs* untreated cells.

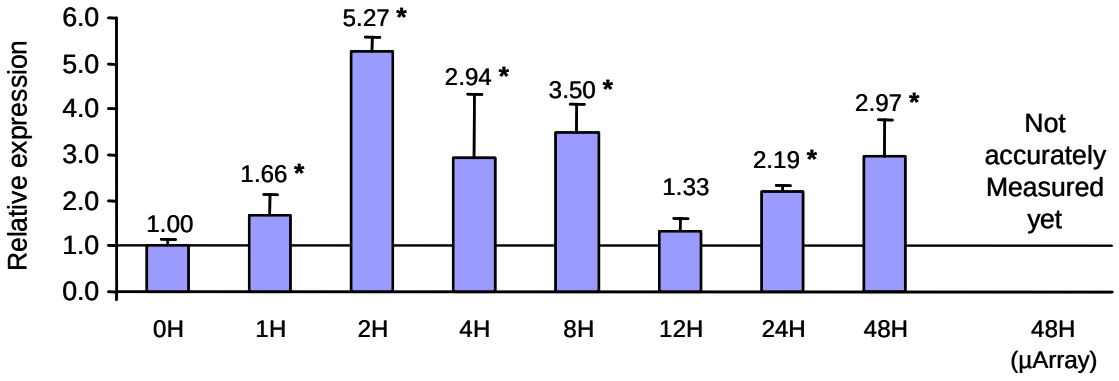
TSP1 transcription is quickly upregulated by TGF-β during the first 12-24H. However it appeared on the melt curve of the PCR that with the primers THBSP1-A a second peak (t° 85°C) is appearing in the reaction after 48H (graph next page). This peak seems smaller in TGF-β treated cell samples than in untreated samples. This peak correspond to an amplification product of ≈250bp which should be sequenced. It may be possible that it is a splicing variant repressed by TGF-β, however no alternative splicing has been detected so far for TSP1.

TSP1 is known as an activator of TGF-β (from the latent to the active form; Crawford *et al.* 1998. Cell. 93:1159) and was characterized as an inhibitor of angiogenesis (Good *et al.* 1990. PNAS. 87:6624) or of metastasis (Volpert *et al.* 1998. PNAS. 95:6343). However breast or prostate tumors can become resistant to TSP1 anti-angiogenic properties (Filleur *et al.* 2001. Genes & Dev. 15:1373; Fontana *et al.* 2005. Int. J. Cancer. 116:686). It is possible that TGF-β is involved in the acquisition of this resistance.

Link to PubMed Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=7057



IL-11

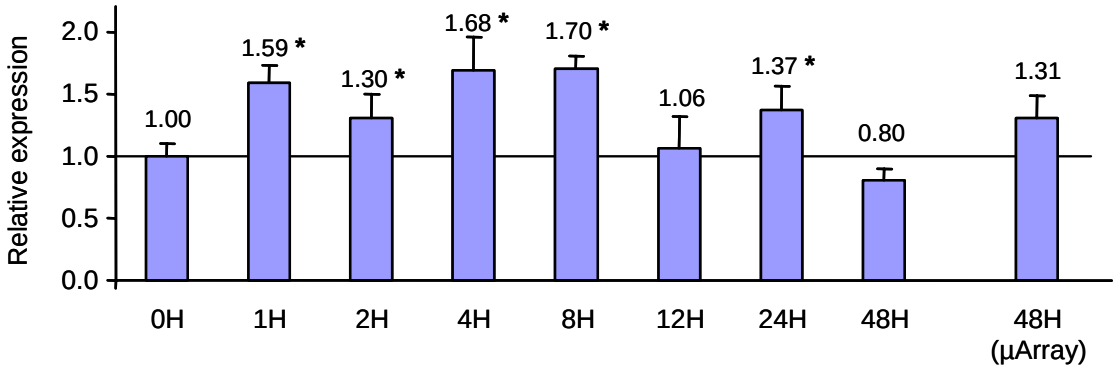


No changes detected by the micro-array.

IL-11 transcription is transiently increased by TGF-β (maximum at 2H). A secondary activation may also occur, starting at 24H. TGF-β control of IL-11 transcription has been very well studied in breast cancer cells (Kang *et al.* 2005. PNAS. 102:13909). They noticed an immediate gene response, peaking at 2H and gradually declining thereafter,

which is well correlated with our results. IL-11 is also known to be involved in osteoclastogenesis and in osteolytic breast cancer metastasis. The transient overexpression induced by TGF-β explain that IL-11 was not pinpointed in the micro-array.

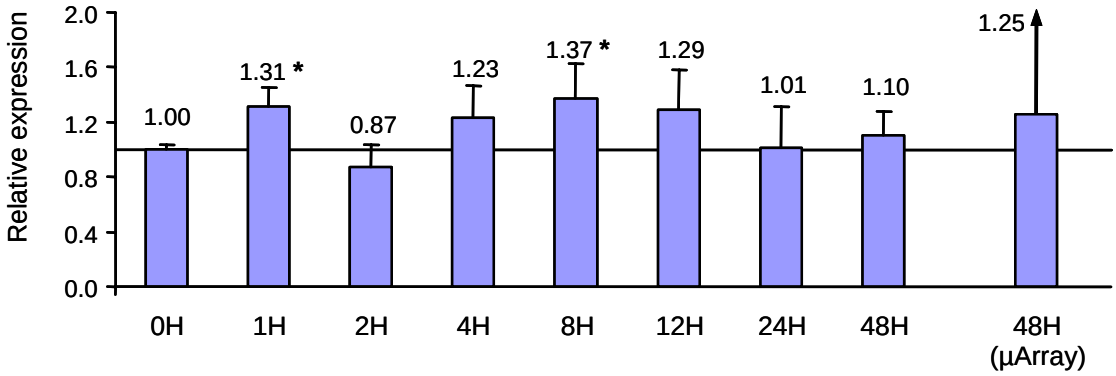
Cyr61 (or CCN1)



No changes detected by the micro-array.

TGF-β induces a slight increase in Cyr61 transcription (maximum ×1.70, at 8H). Cyr61 is known to be a target of TGF-β, and Bartholin *et al.* (Cancer Letters 2006) fully described the effects of TGF-β in this promoter. These results are consistent with findings in MDA-MB-231 (transient activation, maximum about ×1.80, at 1H).

PTHrP

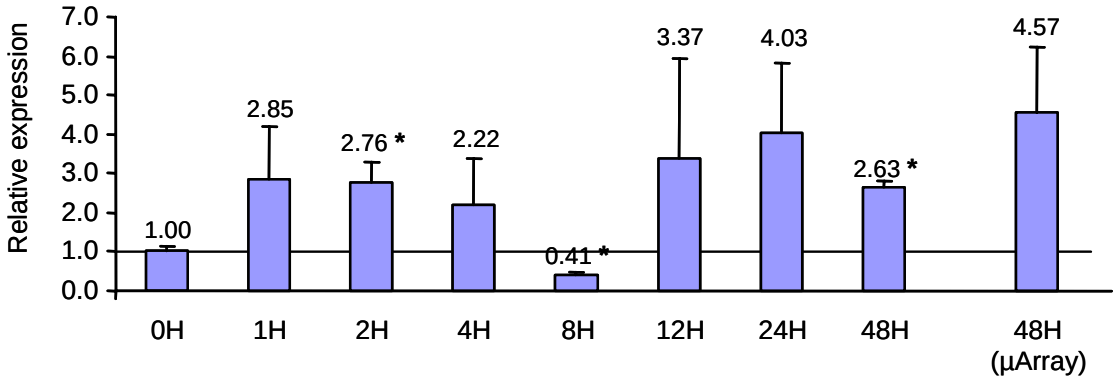


Fold change measured by the micro-array: ×3.31 or ×3.04 (pending on the probe) *vs* untreated cells.

PTHrP secretion has been known for a long time to be increased by TGF-β and to be very important for bone metastasis osteolysis. However, this quantification by real-time PCR could not detect any changes of the transcription induced by TGF-β. According to the Ct values observed, PTHrP, among the different genes assessed seems to be one of the most expressed. It is then possible that the transcription can not be further increased. The higher secretion could then be due to a higher stability of the mRNA or to regulation of the translation.

Treatment with actinomycin D after TGF-β treatment may then be realized to assess the degradation rate of PTHrP mRNA.

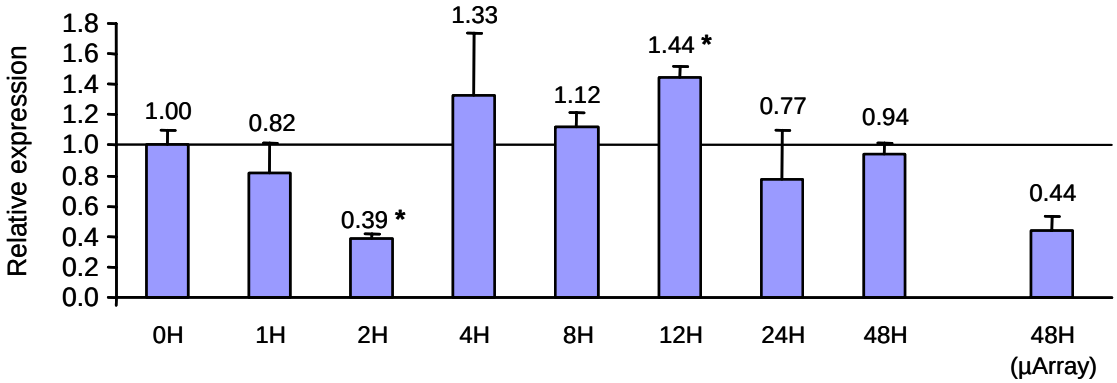
CTGF (or CCN2)



Fold change measured by the micro-array: ×2.93 *vs* untreated cells.

No statistically different variations of CTGF mRNA production were detected in PC-3 cells after TGF-β treatment. This is quite surprising since CTGF is known to be a target gene of TGF-β. We will survey other prostate cancer cells with respect to the effects of TGF-β on CTGF transcription.

IL-6

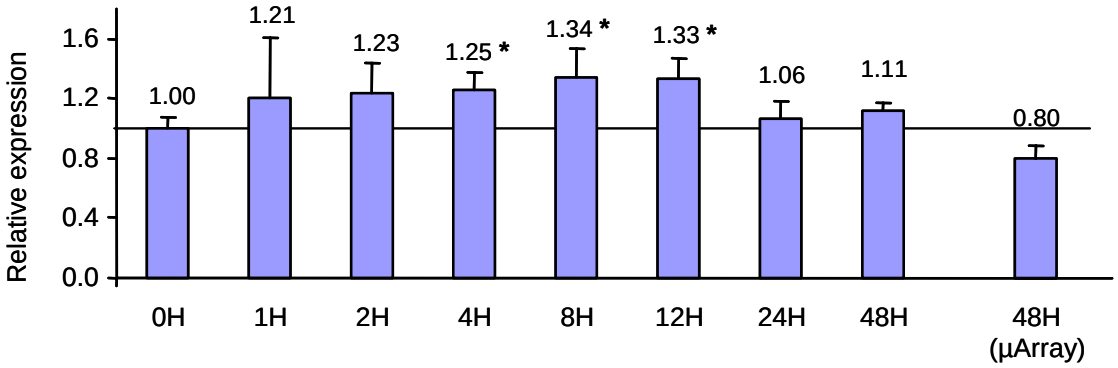


No changes detected by the micro-array.

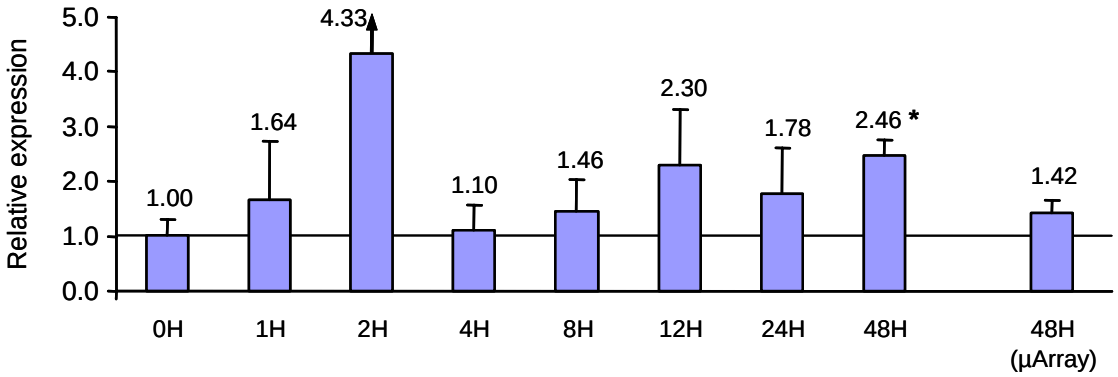
TGF-β is known to increase IL-6 production in PC-3 cells, based on ELISA assay. However no significant changes of the transcription were observed by qRT-PCR. This increase maybe due to mRNA stabilization or increased translation, as for PTHrP.

VEGF

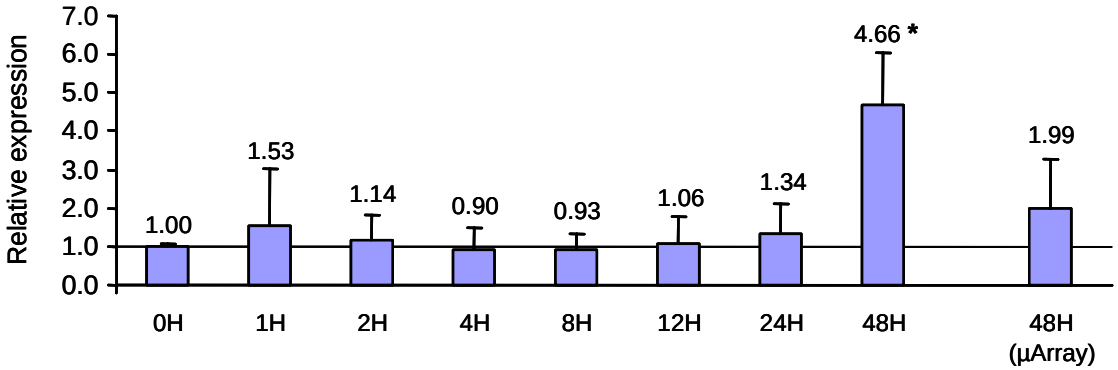
VEGF all isoforms



VEGF121



VEGF165



Fold change measured by the micro-array: ×2.29 *vs* untreated cells.

As opposed to what is suggested by the micro-array, no big increase of VEGF transcription was detected by qRT-PCR in PC-3 cells, after TGF-β treatment. However, we know that the VEGF protein secretion is increased by TGF-β. It was then possible that only some isoforms of VEGF were affected, we assessed then the effects of TGF-β on VEGF121 (or VEGF-F) and VEGF165 (or VEGF-D), expressed in PC-3. However no significant effect of TGF-β was observed on these mRNA.

Another assay of VEGF165 mRNA, at 48H, was realized with a higher amount of cDNA as template and showed no effect of TGF-β on the transcription of this isoform. Effects of TGF-β on VEGF may be due to regulation downstream of the transcription.

Reportable Outcomes

Presentations:

- Cancer and Bone. Meet the Professor Session, American Society for Bone and Mineral Research Meeting, Seattle, Washington, September, 2004
- Endothelin A receptor blockade and bisphosphonate therapy in prostate cancer bone metastases. Prostate Cancer Foundation Scientific Retreat (formerly CAPCURE), Lake Tahoe, NV, October, 2004
- Molecular mechanisms of bone metastases. National Cancer Institute, NIH, November, 2004
- TGFβ blockade in bone metastases. Biogen Advisory Board, Cambridge, MA, December, 2004
- Molecular mechanisms of bone metastases. Endocrinology Grand Rounds, NIH, December, 2004
- Bone metastases: molecular mechanisms and therapeutic interventions. Visiting Professor, Johns Hopkins Cancer Center, February, 2005
- Molecular mechanisms of osteoblastic bone metastases. Orthopaedic Research Society Meeting, Washington DC, February, 2005
- Blockade of TGFβ signaling in breast cancer metastases to bone. TGFβ Keystone Meeting, Keystone, CO, March, 2005
- Endothelin-1 in osteoblastic bone metastases: mechanisms and therapeutic implications. Experimental Biology Meeting, San Diego, CA April 2005
- Mechanisms of osteoblastic bone metastases. IVth North American Symposium skeletal complications of malignancy. NIH/NCI, Bethesda, MD, April 2005
- Mechanisms of osteolytic metastases to bone: Implications for therapy. Visiting professor, Fox Chase Cancer Center, Philadelphia, PA, May 2005
- Role of TGFβ in breast cancer metastases to bone. Seminar, Serono, Boston, MA, May 2005
- Molecular Mechanisms of Osteoblastic Metastases: Implications for therapy. Prostate Cancer: Road Map to the Future. Roswell Park Institute Sponsored Symposium in Niagra Falls NY, July 2005.

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Conclusions

A central tenet of the field of bone metastases is that the bone microenvironment supplies factors, such as TGF-β, stimulating prostate cancer cell signaling and altering their phenotype. TGF-β signaling in cancer is however complex and can lead to the activation of numerous genes. We have identified many of these genes by microarray analysis and have validated the gene reported here. Of these, PMEPA1 as the most highly upregulated gene. We have cloned the PMEPA1 promoter and full-length gene and have begun promoter analysis of the TGFβ response element. We are in the process of overexpressing PMEPA1 in prostate cancer lines. In vivo experiments are in progress to determine the effect of a TGFβ RI kinase inhibitor, SD-208, on the development and progression of prostate cancer metastases to bone due to PC-3, LuCAP and C42B prostate cancers.

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